THE JOURNAL OF INFECTIOUS DISEASES • VOL. 136, SUPPLEMENT • AUGUST 1977

Mechanisms of Disease and Immunity in Cholera: A Review

Jan Holmgren and Ann-Mari Svennerholm

From the Institute of Medical Microbiology, University of Göteborg, Göteborg, Sweden

The adenyl cyclase-activating enterotoxin of *Vibrio cholerae* was shown to contain two types of subunit: six smaller units (L) that are responsible for the binding to cell membrane receptors and a larger unit (H) that mediates the toxic action. The receptor was identified as the ganglioside G_{M1} (galactosyl-N-acetylgalactosaminyl [sialosyl] lactosyl ceramide), and the results suggested that penetration of the toxin molecule into the membrane follows the rapid binding to G_{M1}. The relationship of these findings to the mechanism of protective immunity, which is mediated by antibodies to the enterotoxin as well as those to the cell wall lipopolysaccharide of *V. cholerae*, was investigated. The antitoxic antibodies were directed mainly against the L subunit and protected by preventing binding of toxin; the antibacterial antibodies probably interfered with adhesion of *V. cholerae* to the intestine. The finding that the immune responses to toxin and bacteria act synergistically in protection against experimental cholera indicates that an improved cholera vaccine should contain both toxoid and lipopolysaccharide as antigens. In the rabbit, either subcutaneous or enteral immunization gave rise to intestinal synthesis of specific antibodies to *V. cholerae*.

Unlike *Shigella* and *Salmonella*, which cause disease after penetration into or across the intestinal epithelium, *Vibrio cholerae* is strictly noninvasive. The essential pathogenic events in cholera include (*1*) ingestion of cholera vibrios with contaminated water or food, (*2*) entrance of viable bacteria into the small bowel, (*3*) bacterial multiplication and production of an exotoxin that acts on the intestinal epithelium, and (*4*) hypersecretion of isotonic fluid from the enterotoxin-stimulated small bowel.

Pathogenesis

Adhesion of V. cholerae. Gastric acidity has been found to play an important role in determining the minimal infective dose of *V. cholerae*. This relationship was quantitated in studies by Cash et al. [1], who found that 10^{11} live vibrios were usually required to produce disease in volunteers when the bacteria were given without bicarbonate. In contrast, a dose of 10^6 organisms was effective when administered together with 2 g of $NaHCO_3$. Vibrios that survive the hostile environment in the stomach must be capable of living in the small bowel and of multiplication and production of toxin. Penetration through the mucous layer and adhesion to the epithelium may be the mechanisms by which *V. cholerae* escape the intestinal cleaning brought about by peristalsis (figure 1). La Brec et al. [2] found that in infected guinea pigs *V. cholerae* covered the mucosa of the small bowel in a manner suggesting adhesion to the intact epithelium of the villi. This observation was extended by Freter [3], who found that the adherence of *V. cholerae* to the intestinal mucosa of the rabbit withstood washing. Williams et al. [4] have stressed the importance of the motility of *V. cholerae* in the penetration of the mucous layer that covers the intestinal epithelium. The mucinases of *V. cholerae*, which were once believed to be the actual choleragenic factors, may play a role in this process.

Intestinal secretion. The pathogenesis of cholera is closely related to the action of the *V. cholerae* enterotoxin on the small intestine. Strong evidence indicates that active secretion of electrolytes by the enterotoxin-stimulated epithelium accounts for the loss of fluid consequent to the ability of cholera toxin to increase the levels of adenosine 3′:5′ cyclic monophosphate (cyclic AMP) in

The experimental work described was supported financially by grant no. 16 X-3382 from the Swedish Medical Research Council and by grants from the World Health Organization.

Requests for reprints should be addressed to Dr. Jan Holmgren, Institute of Medical Microbiology, University of Göteborg, Guldhedsgatan 10, S-413 46 Göteborg, Sweden.

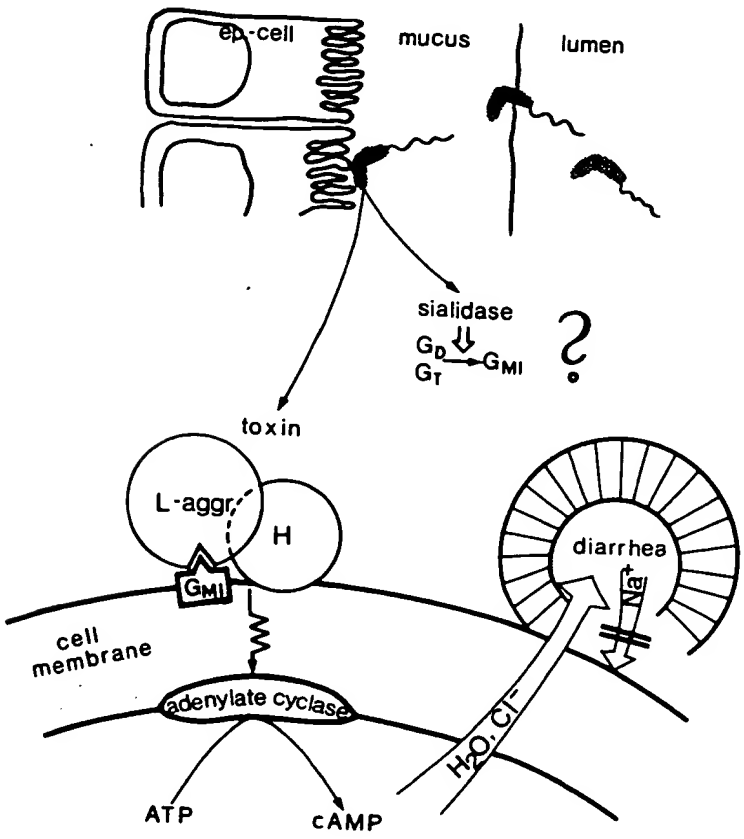

Figure 1. Postulated pathogenic mechanisms in cholera. *Top*, penetration of *Vibrio cholerae* through the mucous layer and adhesion to the intestinal epithelium (ep.) *Center*, a possible receptor-creating role of sialidase (neuraminidase). *Bottom*, stimulation of intestinal hypersecretion by enterotoxin. L-aggr = aggregated L subunits; see text for definition of other abbreviations.

the mucosal cells [5]. The key observations that clarified the link between the action of cholera toxin, cyclic AMP, and intestinal secretion were provided by Field [5] and his associates. Using a system in which ion fluxes across the intestinal epithelium could be measured, they demonstrated that, when exposed to cyclic AMP or theophylline (which inhibits the cyclic AMP-degrading phosphodiesterase system), the epithelium responded with net secretion of chloride and inhibition of sodium absorption. Identical effects on these ion fluxes were observed, after a lag period, by instillation of cholera toxin on the lumen side of the intestine. Several groups demonstrated elevated levels of cyclic AMP in the mucosa of the small bowel during clinical as well as experimental cholera. Using the purified toxin [6], Sharp and Hynie [7] and Kimberg et al. [8] showed that the enterotoxin of *V. cholerae* exerted its effect on intestinal cyclic AMP by activating the adenyl cyclase system in the epithelial cells.

Subunit structure of cholera toxin. The structure-function relationship of cholera toxin, the nature of the intestinal cell membrane receptor, and the mechanism of activation of adenyl cyclase have been studied in our laboratory. Different strains of *V. cholerae*, belonging to either of the two major serotypes (Inaba and Ogawa), were shown to produce an immunologically identical toxin [9]. Our studies revealed that cholera toxin contains two types of noncovalently linked subunits [10]. These subunits could be separated either by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate and urea or by gel filtration in acidic glycine-urea buffer. The molecular weights of the two subunits were estimated to be 28,000 and ~8,000 daltons, respectively; we called these subunits H (heavy) and L (light). Alternative designations are A and B subunits, respectively. We concluded that each toxin molecule, which has a molecular weight of 84,000 daltons, consists of one H subunit and about six L subunits. We also observed that the H subunit contains two peptide chains that can be sepa-

rated by reduction and alkylation [10]. Finkelstein isolated, in addition to the active toxin, a nontoxic protein, choleragenoid, that is immunologically similar to the toxin but has a molecular weight of only 56,000 daltons [6]. We found that this protein contains the same number of L subunits as does the toxin but that it lacks the H subunit; this finding indicated a critical role of the latter subunit for the biologic activity of cholera toxin. Our model of the subunit structure of cholera toxin has subsequently been confirmed by studies of Cuatrecasas et al. [11], S. van Heyningen [12], and others.

Structure of toxin receptor. The first event in the action of cholera toxin on the intestinal cells is a rapid, tight binding to the cell membrane. Prompted by the observation by W. van Heyningen et al. [13] that a crude mixture of gangliosides could inactivate cholera toxin, we investigated, in collaboration with Dr. L. Svennerholm (University of Göteborg, Göteborg, Sweden), whether a ganglioside might constitute or be part of the membrane receptor. We found that the pure monosialosylganglioside G_{M1} (galactosyl-N-acetylgalactosaminyl[sialosyl] lactosyl ceramide) bound and inactivated cholera toxin in a 1:1 molar ratio, whereas no other glycolipid reacted in a specific manner with cholera toxin [14]. Further experiments indicated that the number of binding sites for cholera toxin on different cells was directly correlated to the amount of G_{M1} in the cell membrane [15]. Chemical modification of various amino acid residues in cholera toxin concomitantly affected binding to cells and to matrix-coupled G_{M1} [16]. Furthermore, in agreement with results of studies of fat cells by Cuatrecasas [17], we found that G_{M1} could be incorporated into the membrane of intestinal cells. Such incorporation specifically increased the number of binding sites for cholera toxin in the intestine and also enhanced the susceptibility of the intestine to the diarrheagenic action of the toxin [15]. This accumulated evidence strongly indicates that the ganglioside G_{M1} is the membrane receptor for cholera toxin.

Mechanism of action of toxin. Studies with the isolated L and H subunits and with toxin derivatives containing various proportions of both L and H provided knowledge about the respective pathogenic roles of the two types of subunits in cholera toxin (figure 1). Subunit L was found to mediate the binding of toxin to the cell membrane but not the activation of adenyl cyclase. The H subunit could not bind to cells but was essential for biologic activity. We concluded that the role of the L subunit-G_{M1} receptor reaction is to facilitate productive interaction of the active subunit H with the cell [18].

Our recent studies have shown that, within a few minutes after the reversible binding of toxin to the cell surface receptors, the toxin becomes irreversibly associated with the cell, probably by penetration into the membrane [16]. The results of our work also indicated that, in addition to G_{M1} and adenyl cyclase, cell structures are involved in the activation process [16]. However, the exact mechanism by which subunit H (or one of its peptides) mediates stimulation of adenyl cyclase remains undefined. One possibility is that it acts intracellularly, in which case the action is probably enzymatic, since only a very small amount of toxin can be demonstrated in the cytosol. Another possibility is that the H subunit, either enzymatically or allosterically, modifies adenyl cyclase without leaving the membrane.

Neuraminidase of V. cholerae. Cholera vibrios produce neuraminidase, which can hydrolyze oligosialosylgangliosides (primarily G_{D1} [disialosyl galactosyl-N-acetylgalactosaminyllactosyl ceramide and G_{T1} [trisialosylgalactosyl-N-acetylgalactosaminyllactosyl ceramide]) to G_{M1}. Therefore, we expected to find a pathogenic role of this enzyme in cholera as a means by which the vibrios could increase the number of toxin receptors on intestinal cells (figure 1). G_{D1} and G_{T1} isolated from small bowel mucosa were completely hydrolyzed to G_{M1} by incubation with neuraminidase from *V. cholerae*. However, we have found that the neuraminidase appears to be unable to act on the intact intestinal epithelium or on its dispersed cells. Thus, the ganglioside pattern, the number of binding sites for cholera toxin, and the sensitivity to the diarrheagenic action of toxin were not influenced by neuraminidase [15].

Immunology

Observations, in cholera-endemic areas, of a drastic fall in the incidence of cholera with age, a low frequency of reinfection, and a reduction of the disease by vaccination indicate the significance of

acquired immunity in cholera [19]. All available data suggest that this immunity is due to humoral antibodies, and there are no indications of protective cellular immune mechanisms. Clinical cholera gives rise to antibodies to the bacteria as well as to the enterotoxin. A protective role of both of these types of antibodies has been demonstrated in experimental animals. In humans the enhanced resistance to cholera that is induced by vaccination with somatic antigen indicates a protective function of antibacterial immunity. The significance of antitoxic antibodies for protection against natural disease remains to be established. However, preliminary data from the 1974–1975 field trial in Bangladesh suggest that a glutaraldehyde-prepared cholera toxoid can give rise to brief protection [20].

Antibacterial immunity. Purified lipopolysaccharide (LPS) of *V. cholerae* has been found to induce significant protection against cholera in both humans and experimental animals. Our studies suggest that the antibacterial protective immunity is exclusively mediated by antibodies to the LPS. Thus, we have found that antibodies to LPS, purified from hyperimmune sera to whole vibrios by affinity chromatography, possess the entire protective capacity of the sera from which they are derived [21]. Consequently, we have not been able to substantiate the observation of Neoh and Rowley [22] of a protective role of antibodies to a cell envelope protein. However, the possibility cannot be excluded that this antigen might have been present in the minor protein fraction that could not be eliminated from our LPS preparations despite repeated extraction with phenol-water.

The LPS of Ogawa and Inaba strains of *V. cholerae* cross-react immunologically through the antigenic determinant A but are differentiated by the presence of the type-specific determinants B and C, respectively. In field trials, monovalent vaccines (i.e., Inaba or Ogawa) have induced considerably better protection against infection with homologous strains than against infection with heterologous strains of *V. cholerae*. In experimental studies we have found that, in relation to their amount, the type-specific and the cross-reactive antibodies have similar protective capacity, a finding indicating that only the higher titer of type-specific antibody is responsible for the superior homologous protection (figure 2).

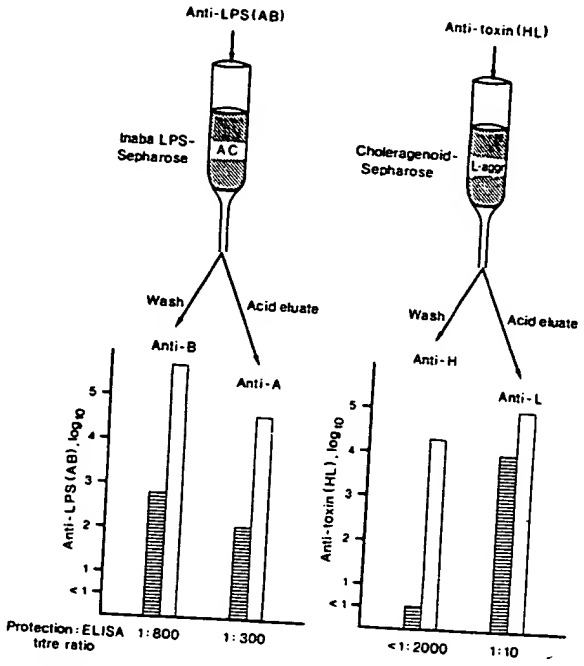

Figure 2. Evaluation of relative protective capacities of antibodies to different determinants on lipopolysaccharide (LPS) and enterotoxin of *Vibrio cholerae*. *Left*: Hyperimmune serum to *V. cholerae* Ogawa LPS (A and B determinants) was applied to a column with Inaba LPS covalently coupled to Sepharose. Unattached type-specific (anti-B) antibodies were recovered by washing of the column with 0.05 M phosphate buffer (pH 7), and specifically bound Inaba-shared (anti-A) antibodies were isolated by subsequent elution with 1 M acetate buffer (pH 3). Amounts of antibody were estimated with an enzyme-linked immunosorbent assay (ELISA; open bars), and protection against challenge with live Ogawa organisms was studied in the rabbit small intestine (hatched bars). *Right*: Antibodies to enterotoxin subunits L and H (light and heavy, respectively) were separated on a choleragenoid-Sepharose column by a procedure analogous to that described for anti-LPS B and A antibodies. Their protective capacities were titrated with use of an intradermal neutralization assay. AC = antigenic determinants on Inaba LPS; L-aggr = aggregated L subunits.

Antitoxic immunity. We have observed that immunization with highly purified cholera toxin gives rise to a somewhat higher degree of protection against experimental cholera than does a corresponding immunization with choleragenoid. The greater efficiency of the active toxin could be due to its ability to enhance nonspecifically various immune responses [23], perhaps including immunity to itself. Its efficiency could also be due to a protective role of antibodies induced against the

"toxic" H subunit. We tried to evaluate the relative protective capacity of antitoxins directed to subunits L and H, respectively. Antibodies to the H subunit were obtained by passing antiserum to whole toxin through a column containing choleragenoid covalently coupled to Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Antibodies to the L subunit, bound by the column, were isolated by subsequent elution with acidic buffer. With all sera tested, antibodies to the L subunit were not only present in considerably larger quantities than were antibodies to the H subunit but also had, in relation to amount, a much higher toxin-neutralizing capacity (figure 2).

Mode of action of protective antibodies. Since neither the cholera vibrios nor their enterotoxin appear to penetrate the intestinal mucosa, only those antibacterial and antitoxic antibodies present within the intestinal lumen or at the mucosal surface can be expected to be effective. Our observation that antitoxic immunity is almost exclusively mediated by antibodies to the L subunit indicates that the protective antitoxic antibodies act by preventing binding of toxin to the G_{M1} ganglioside receptor in the intestinal epithelium rather than by interacting with the "toxic" site of the H subunit. Studies with separated immunoglobulin fractions showed that IgG antibodies have a much greater neutralizing potency than do antibodies of the IgM class, which are practically without effect [24]. A protective role of IgA antibodies has been documented by Kaur et al. [25], who extracted secretory IgA antibodies with toxin-neutralizing capacity from crypts of the intestinal mucosa of immunized rabbits.

The mode of action of the protective antibacterial antibodies is less clear. Neither bacterial killing nor opsonization, which are the common pathways by which antibodies mediate protection against bacterial infections, appear to be significant in combatting *V. cholerae* in the small bowel. Instead, Freter [3] has suggested that the antibacterial antibodies act by inhibiting the adhesion of *V. cholerae* to the mucosal surface, thus enhancing expulsion of vibrios by peristalsis and impairing delivery of toxin. This concept is based on the finding that passive immunization with specific antibacterial antiserum placed in the intestine, as well as active immunization with killed vibrios, resulted in a marked reduction of the number of vibrios adsorbed to the mucosa of infected intestinal loops but did not affect multiplication of *V. cholerae*. The possibility that antibacterial antibodies also impair enterotoxin production has not been excluded. Irrespective of the mechanism of action, IgG, IgA, and IgM antibodies to *V. cholerae* are all capable of mediating protection if they are present in the intestine. In a recent study Steele et al. [26] found that antibodies of these three classes were equally effective in preventing experimental cholera in mice.

Origin of protective antibodies. Although antibodies to *V. cholerae* must exert their action within the intestine, the site of synthesis may still be outside the intestine. Numerous studies have demonstrated a significant influx of serum immunoglobulin into the small bowel, an observation suggesting that protective antibodies to *V. cholerae* may be derived from the circulation. Consistent with this possibility, an inverse correlation has been found in cholera-endemic areas between titers of vibriocidal antibody in sera and the incidence of cholera [19]. After parenteral immunization there was a direct correlation between the titers of antitoxin in sera and protection against experimental cholera in dogs [27] and rabbits [28]. Furthermore, transfusion experiments by ourselves and others have indicated that serum antibodies can mediate protection against experimental cholera.

The importance of local formation of antibody in the intestine was first indicated by Burrows et al. [29], who observed protection against experimental cholera in the absence of significant titers of antibody in serum after vaccination with killed vibrios. Later investigations demonstrated that protective immunity against enterotoxin can be induced, with formation of only a small amount of serum antibody, by means of local administration of antigen [28]. Not until recently were methods permitting direct quantitation of local antibody formation in tissues used in studies of immunity to cholera. Using a toxin antigen-specific immunofluorescence method, Pierce and Gowans [30] observed that repeated local administration of a formalin-prepared cholera toxoid gave rise to specific antibody-containing cells in the lamina propria of the small intestine of the rat.

We have quantitated local as well as systemic antibody formation to cholera antigens after various routes of immunization by allowing tissue

specimens from immunized rabbits to synthesize radiolabeled proteins in vitro and by then isolating the newly produced specific antibodies by affinity chromatography. We found that two sc vaccinations with a mixture of *V. cholerae* LPS and enterotoxin induced significant synthesis of radiolabeled antibodies to both antigens not only in the spleen but also in intestinal tissue and Peyer's patches [31]. Repeated instillation of antigen into the intestinal lumen induced synthesis of similar amounts of radiolabeled antibodies in the intestine as after parenteral immunization but of smaller amounts in the spleen; the titers of antibody in serum were much lower than after sc vaccination. Studies of antibodies in the incubation medium using an immunoglobulin class-specific, enzyme-linked immunosorbent assay (ELISA) [24] showed that IgG predominated in spleen medium regardless of route of immunization. In intestinal medium from rabbits immunized sc or enterally (large doses), the titers of IgG were similar to or higher than the titers of IgA, whereas after enteral immunization with a smaller dose of antigen, the titers of IgA were higher (table 1). In view of the similar or even greater intestinal antibody response after enteral than after sc immunization, it is notable that the parenteral route gave a higher level of protective immunity against intestinal challenge with live vibrios as well as with toxin (table 2) This fact probably reflects a significant contribution of serum antibodies, at least in the rabbit model.

Design of improved cholera vaccine. The limited efficacy of killed vibrio vaccines and the recognition of the critical role of the immunogenic enterotoxin in the pathogenesis of cholera have prompted intense efforts to develop an improved cholera vaccine based on toxoid. Recent studies in our laboratory have indicated that an optimal cholera vaccine should be of the combined type, containing toxoid as well as somatic antigen of *V. cholerae* [32]. Thus, we observed that a combination of *V. cholerae* toxin and LPS antigens induced a more than 100-fold higher degree of immunity in the rabbit against challenge with live vibrios than did immunization with either of the two antigens alone (figure 3). The synergistic effect was not due to enhancement, by an adjuvant action, of the antibacterial and antitoxic immune responses in themselves, i.e., the resistance to challenge with toxin was not enhanced above the response induced by immunization with toxin alone. Instead, the effect apparently resulted from the interference with two separate events in the pathogenesis of cholera.

Table 1. Titers of antibodies in sera of rabbits immunized with lipopolysaccharide (LPS) and toxin of *Vibrio cholerae* and of antibodies in medium from in vitro incubation of tissues from immunized rabbits.

Immunization, antigens*	ELISA titers†				
		Spleen medium		Intestinal tissue medium	
	Serum	IgG	IgA	IgG	IgA
Subcutaneous					
LPS	500,000	300	130	200	200
Toxin	500,000	300	30	80	40
Intraluminal (high dose)					
LPS	80,000	110	30	80	70
Toxin	25,000	160	15	170	90
Intraluminal (low dose)					
LPS	10,000	60	10	20	50
Toxin	3,000	50	20	60	110
Controls					
LPS	100	<10	<10	<10	<10
Toxin	40	<10	<10	<10	<10

*Two equal doses of antigen were administered two weeks apart; each sc injection consisted of 1 mg of LPS and 10 μg of toxin, and the intraluminal instillations consisted of 10 mg of LPS and 330 μg of toxin (high dose) or 2 mg of LPS and 100 μg of toxin (low dose).

†Titers of antibody were measured by the enzyme-linked immunosorbent assay (ELISA) [24].

Table 2. Protection against experimental cholera in the rabbit after various routes of immunization with toxin and lipopolysaccharide of *Vibrio cholerae*.

Immunization*	Protection against†	
	Toxin	Live vibrios
Subcutaneous	>2.8	>160
Intraluminal		
High dose	2.1	80
Low dose	1.6	14

*Immunizations were performed as in table 1. The animals were challenged in the intestine five days after the second immunization with graded doses of crude cholera toxin or live vibrios of strain 569B.

†Protection is expressed as the ratio of the 50% effective dose for immunized animals to that for concurrently tested control animals.

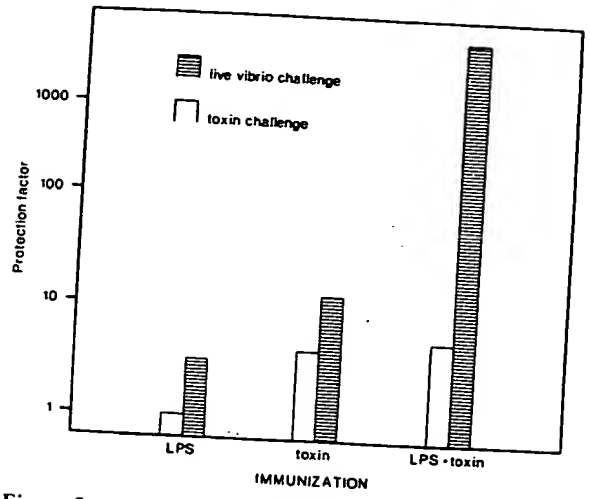

Figure 3. Synergistic protective effect of immunization with *Vibrio cholerae* lipopolysaccharide (LPS) and enterotoxin in combination. Rabbits were given two sc injections two weeks apart and were challenged intraintestinally five days after the second immunization. The immunization doses were 1.25 mg of LPS and 15 μg of choleragen per injection. Protection factors represent ratios of the 50% effective dose for immunized animals to that for concurrently tested control animals.

With regard to the toxoid component of a combined cholera vaccine, traditional methods of inactivation, such as treatment with formalin or glutaraldehyde, have so far met with limited success because of reversion to toxicity and extensive destruction of antigenicity. The recent insight into the relationships between structure, biologic activity, and antigenicity of the enterotoxin molecule suggests at least two possibilities for more specific inactivation of cholera toxin. Preparation of pure subunit L and appropriate reaggregation of this subunit may yield an almost optimal toxoid, since the elimination of subunit H excludes reversion to toxicity without any significant loss of protective antigenic determinants. Alternatively, selective chemical modification of the toxic site in situ by amino acid-specific reagents may be useful. This was indicated in a recent study in which arginyl-specific reagents completely abolished the biologic activity without inhibiting the antibody-fixing capacity of cholera enterotoxin [33].

With regard to the somatic antigen component, the purified bacterial LPS will probably be as effective as the whole vibrios. Ultimately, it might be possible to isolate, and perhaps synthesize, the immunodeterminant fragments of the LPS molecule. Such fragments, having the advantage of being free from endotoxic activity, probably must be coupled to a carrier to be immunogenic. Use of cholera toxoid as carrier for the somatic antigen component may yield an attractive combined vaccine.

References

1. Cash, R., Music, S., Libonati, J., Snyder, M., Wenzel, R., Hornick, B. Response of man to infection with *V. cholerae*. I. Clinical, serologic, and bacteriologic responses to a known inoculum. J. Infect. Dis. 129:45–52, 1974.
2. La Brec, E. H., Sprinz, H., Schneider, H., Formal, S. B. Localization of vibrios in experimental cholera: a fluorescent antibody study in guinea-pigs. *In* O. A. Bushnell and C. S. Brookhyser [ed.]. Proceedings of the Cholera Research Symposium, Honolulu, Hawaii, 1965. U.S. Public Health Service publication no. 1328, 1965, p. 272–276.
3. Freter, R. Studies of the mechanism of action of intestinal antibody in experimental cholera. Tex. Rep. Biol. Med. 27 (Suppl. 1):299–316, 1965.
4. Williams, H. R., Jr., Verwey, W. F., Schrank, G., Hurry, E. K. An in vitro antigen-antibody reaction in relation to a hypothesis of intestinal immunity to cholera. Proceedings of the Ninth Joint Conference of the U.S.-Japan Cooperative Medical Science Program, 1973. U.S. Department of State publication no. 8762, 1974, p. 196–213.
5. Field, M. Intestinal secretion of cyclic AMP and its role in cholera. N. Engl. J. Med. 284:1137–1144, 1971.
6. Finkelstein, R. Cholera. CRC Crit. Rev. Microbiol. 2: 553–623, 1973.
7. Sharp, G. W. G., Hynie, S. Stimulation of intestinal adenyl-cyclase by cholera toxin. Nature [Lond.] 229: 266–269, 1971.
8. Kimberg, D. V., Field, M., Johnson, J., Henderson, A., Gershon, E. Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. J. Clin. Invest. 50:1218–1230, 1971.
9. Holmgren, J., Lönnroth, I., Ouchterlony, Ö. Immunochemical studies of two cholera toxin-combining standard culture filtrate preparations of *Vibrio cholerae*. Infec. Immun. 3:747–755, 1971.
10. Lönnroth, I., Holmgren, J. Subunit structure of cholera toxin. J. Gen. Microbiol. 76:417–427, 1973.
11. Cuatrecasas, P., Parikh, I., Hollenberg, M. D. Affinity chromatography and structural analysis of *Vibrio cholerae* enterotoxin-ganglioside agarose and the biological effects of ganglioside-containing soluble polymers. Biochemistry 12:4253–4264, 1973.
12. van Heyningen, S. Cholera toxin: interaction of subunits with ganglioside G_{M1}. Science 183:656–657, 1974.

13. van Heyningen, W. E., Carpenter, C. C. J., Pierce, N. F., Greenough, W. B. III. Deactivation of cholera toxin by ganglioside. J. Infect. Dis. 124:415–418, 1971.
14. Holmgren, J., Lönnroth, I., Svennerholm, L. Tissue receptor for cholera exotoxin: postulated structure from studies with G_{M1} ganglioside and related glycolipids. Infec. Immun. 8:208–214, 1973.
15. Holmgren, J., Lönnroth, I., Månsson, J-E., Svennerholm, L. Interaction of cholera toxin and membrane G_{M1} ganglioside of small intestine. Proc. Natl. Acad. Sci. U.S.A. 72:2520–2524, 1975.
16. Holmgren, J., Lönnroth, I. Cholera toxin and the adenylate cyclase activating signal. J. Infect. Dis. 133 (Suppl.):S64–S74, 1976.
17. Cuatrecasas, P. Gangliosides and membrane receptors for cholera toxin. Biochemistry 12:3558–3566, 1973.
18. Holmgren, J., Lindholm, L., Lönnroth, I. Interaction of cholera toxin and toxin derivatives with lymphocytes. I. Binding properties and interference with lectin-induced cellular stimulation. J. Exp. Med. 139: 801–809, 1974.
19. Mosley, W. H. The role of immunity in cholera. A review of epidemiological and serological studies. Tex. Rep. Biol. Med. 27 (Suppl 1):227–241, 1969.
20. Curlin, G., Levine, R., Aziz, K. M. A., Rahman, M., Verwey, W. F. Field trial of cholera toxoid. Proceedings of the Eleventh Joint Conference of the U.S.-Japan Cooperative Medical Science Program, New Orleans, 1975. U.S. Department of Health, Education, and Welfare, 1976, p. 313–330.
21. Svennerholm, A-M. Experimental studies in cholera immunization. IV. The antibody response to formalinized *Vibrio cholerae* and purified endotoxin with special reference to protective capacity. Int. Arch. Allerg. Appl. Immunol. 49:434–452, 1975.
22. Neoh, S. H., Rowley, D. The antigens of *Vibrio cholerae* involved in the vibriocidal action of antibody and complement. J. Infect. Dis. 121:505–513, 1970.
23. Northrup, R. S., Fauci, A. S. Adjuvant effect of cholera enterotoxin on the immune responses of the mouse to sheep red blood cells. J. Infect. Dis. 125:672–673, 1972.
24. Holmgren, J., Svennerholm, A-M. Enzyme-linked immunosorbent assays for cholera serology. Infec. Immun. 7:759–763, 1973.
25. Kaur, J., McGhee, J. R., Burrows, W. Immunity to cholera: the occurence and nature of antibody-active immunoglobulins in the lower ileum of the rabbit. J. Immunol. 108:387–395, 1972.
26. Steele, E. J., Chaicumpa, N., Rowley, D. Isolation and biological properties of rabbit antibody to *V. cholerae*. J. Infect. Dis. 130:93–103, 1974.
27. Curlin, G., Craig, J. P., Subong, A., Carpenter, C. C. J. Antitoxic immunity in experimental cholera. J. Infect. Dis. 121:463–470, 1970.
28. Holmgren, J., Svennerholm, A-M., Ouchterlony, Ö., Andersson, Å., Wallerström, G., Westerberg-Berndtsson, U. Antitoxic immunity in experimental cholera: protection and serum and local antibody responses in rabbits after enteric and parenteral immunization. Infec. Immun. 12:1331–1340, 1975.
29. Burrows, W., Elliot, M. E., Havens, I. Studies on immunity to Asiatic cholera. IV. The excretion of copro-antibody in experimental enteric cholera in the guinea pig. J. Infect. Dis. 81:261–281, 1947.
30. Pierce, N. F., Gowans, J. L. Cellular kinetics of the intestinal immune responses to cholera toxoid in rats. J. Exp. Med. 142:1550–1563, 1975.
31. Svennerholm, A-M., Holmgren, J. Immunoglobulin and specific antibody synthesis in vitro by enteral and nonenteral lymphoid tissues after subcutaneous cholera immunization. Infec. Immun. 15:360–369, 1977.
32. Svennerholm, A-M., Holmgren, J. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoids. Infec. Immun. 13:735–740, 1976.
33. Lönnroth, I., Holmgren, J. Protein reagent modification of cholera toxin: characterization of effects on antigenic, receptor-binding and toxic properties. J. Gen. Microbiol. 91:263–277, 1975.

The Journal of Infectious Diseases

Volume 136 Supplement August 1977

JIDAQ 136 Suppl S256 (1977)

Medical Microbiology / Clinical Immunology / Epidemiology

Official Publication of the Infectious Diseases Society of America Published by the University of Chicago Press